The Influence of Gamma-Irradiation on the Formation of Free Radicals and Antioxidant Status of Oregano (*Origanum vulgare* L.)

Jana HORVÁTHOVÁ[1], Milan SUHAJ[1], Martin POLOVKA[1], Vlasta BREZOVÁ[2] and Peter ŠIMKO[1]

[1]Food Research Institute, Bratislava, Slovak Republic, [2]Institute of Physical Chemistry and Chemical Physics, Faculty of Chemical and Food Technology, Slovak University of Technology, Bratislava, Slovak Republic

Abstract

Horváthová J., Suhaj M., Polovka M., Brezová V., Šimko P. (2007): **The influence of gamma-irradiation on the formation of free radicals and antioxidant status of oregano (***Origanum vulgare* L.**)**. Czech J. Food Sci., **25**: 131–143.

The influence of various gamma-radiation dose absorptions on oregano (*Origanum vulgare* L.) solid samples was monitored by means of electron paramagnetic resonance (EPR) spectroscopy. Further, the antioxidant activity of oregano methanol/water extracts was characterised using DPPH (1,1-diphenyl-2-picrylhydrazyl free radical); thiobarbituric acid reactive substances (TBARS); ferric reducing power (FRP); and total content of phenolic compounds (TPC) assays. EPR spectroscopy was used for the investigation of the influence of the absorbed dose on the character of the paramagnetic structures formed, as well as for their thermal stability and life-time characterisation. EPR spectrum of the reference (non-irradiated) sample represents a broad singlet line with unresolved hyperfine splitting, attributable to Mn(II) ions, upon which the additional sharp EPR signal ($g = 2.0022$, $\Delta B_{pp} \sim 1$ mT) is superimposed, assigned to stable semiquinone radicals produced by the oxidation of polyphenolic compounds present in plants. The additional paramagnetic structures of different origin (mostly of cellulose and carbohydrate), possessing diverse thermal stability and life-time, were identified in the gamma-irradiated samples. Immediately after irradiation, a statistically significant increase was observed of the TBARS values and the total content of phenolic compounds in methanol/water oregano extract. The alterations of the antioxidant properties of oregano extracts with the time after the radiation treatment were also monitored. A substantial time-dependent decrease of antioxidant activity was observed, probably as a result of storage, with both irradiated and non-irradiated oregano samples, as obvious from the ferric reducing power test and the content of total phenolic substances. The influence of irradiation and subsequent storage of oregano samples on the DPPH radical-scavenging ability was only negligible.

Keywords: oregano; irradiation; EPR spectroscopy; free radicals; thermal stability; life-time; antioxidant activity

Processing foods with gamma-radiation is now widely accepted as an effective method to maintain the quality of food for a long time. The Directive 1999/3/EC established a Community list of foods and food ingredients that can be treated with ionising radiation and set the maximum average

Supported by the state subprogram "Food – Quality and Safety", No. 2003SP270280E010280E01 and by the Slovak Research and Development Agency, Projects No. APVT-20-004504 (VB) and LPP-0136-06 (MP).

absorbed dose to 10 kGy for dried aromatic herbs, spices, and vegetable seasonings. This is also in accord with the Codex Alimentarius General Standard for irradiated foods, in which the maximum absorbed dose should not exceed 10 kGy, with several exceptions related to specific technological requirements (Codex Stan 2003). The limitations of the Code of Federal Regulation (FDA) restricted the maximum absorbed dose for culinary herbs, seeds, spices, vegetable seasonings, and their blends to 30 kGy (Code of Federal Regulation 2004).

EPR characterisation of irradiated spices

In view of the fact that the gamma-irradiation of foods leads to the production of paramagnetic species, Electron Paramagnetic Resonance (EPR) spectroscopy is considered to be the unique detection technique for their characterisation and investigation.

Among various foods, spices are the most frequently irradiated. Only minor differences were found by Calenberg *et al*. (1998) in EPR spectra of white pepper, sweet red paprika, and nutmeg irradiated either with electron beams or X-rays at the doses from 0 up to 10 kGy. EPR spectroscopy was successfully applied for the study of free radicals in black pepper and the evaluation of the potential of these radicals in identifying the radiation treatment (Franco *et al*. 2004; Polovka *et al*. 2006; Suhaj *et al*. 2006). According to Franco and co-workers a line produced both by radiolysis and thermolysis was observed, and its behaviour upon thermal treatment suggests that it cannot be used as an irradiation marker for the doses up to 30 kGy (Franco *et al*. 2004). On the other hand, as it was concluded by Polovka and co-workers the black pepper samples gamma-irradiated at the doses from 5 to 30 kGy could be reliably recognised also a longer time (up to 6 months) after the radiation treatment, due to different consequences of the temperature growth from 298 to 353K on the EPR signal intensity monitored for non-irradiated and gamma-radiation processed samples (Polovka *et al*. 2006). Two physical methods (viscosimetry with two different ways of the sample preparation and electron spin resonance) were used to detect irradiated black pepper samples also by Formanek *et al*. (1999). As they found, the identification of irradiation in black pepper samples using these methods is accomplishable even after one month of storage at ambient temperature. The thermal behaviour of the organic free radicals induced in irradiated black pepper using EPR spectroscopy was studied by Ukai and Shimoyama (2003). They found that the evolution of free radicals formed in the irradiated pepper obeys a single exponential function and gives a unique time constant. Chabane *et al*. (2001) used thermoluminescence, EPR spectroscopy, and viscosimetric measurements to establish whether or not a spice had been irradiated. Electron spin resonance led to different spectra shapes depending on the chemical composition of the spices and on the type of paramagnetic structure. As they concluded, EPR could only be used as a proof of irradiation for up to several weeks after irradiation, and only with some spices. The EPR method is recommended as a dosimetric detection method for the detection of foods containing cellulose. Additional methods for the detection of irradiated foods are incorporated in several directives and standards, e.g. in EN 13708:2001; EN 1787:2000; EN 13751:2002; EN 13784:2001; EN 1784:1996; EN 1785:1996 and EN 1786:1996.

Antioxidant ability assays

A variety of tests detecting antioxidant ability of food components has been recently suggested. Generally, they can be categorised into two groups: assays for radical scavenging ability, and assays testing the lipid peroxidation inhibition ability under various experimental conditions (Schwarz *et al*. 2001).

As previously mentioned by Antolovich *et al*. (2002), the key factors in the oxidation processes are a proper substrate, an oxidant and an initiator, leading to the formation of various intermediates and subsequent final products. Measurement or monitoring of any of these factors can be used to assess the antioxidant activity.

Scavenging of DPPH is considered to be a valid and easy assay to evaluate the radical-scavenging activity of antioxidants, since the radical compound is stable and need not be generated as it is common in other radical scavenging assays. DPPH is a stable free radical capable to accept an electron from reactive radicals, thus behaving as a radical scavenger (Yordanov 1996). Additionally, DPPH acts as an acceptor of electrons from antioxidants; several electron transfer reactions of DPPH with phenols, amines and other compounds were described in literature (Yordanov 1996;

Staško *et al*. 2002). The depletion of DPPH after the addition of antioxidants can be measured by UV-VIS spectroscopy (λ_{max} = 520 nm), EPR spectroscopy, as well as by other techniques (Polovka *et al*. 2003). It was previously successfully applied e.g. to characterise the antioxidant activities of oregano and black pepper extracts (Schwarz *et al*. 2001; Exarchou *et al*. 2002; Polovka *et al*. 2006; Suhaj *et al*. 2006) or for the test of radical-scavenging ability of several commercial teas and wines (Staško *et al*. 2002, 2006; Polovka *et al*. 2003).

The TBARS (thiobarbituric acid reactive substances) assay is one of the most widely used tests for determining the extent of lipid oxidation. The lipid to be analysed is dissolved in a suitable nonpolar solvent, an aqueous solution of thiobarbituric acid (TBA) is then added to the sample. As a result of the formation of a complex between TBA and secondary – formed aldehydes (although some other secondary reaction products can react with TBA as well), pink colour is developed in the mixture, whose intensity is directly related to the concentration of TBARS in the original sample. It can be effectively determined by measuring its absorbance at 540 nm using a UV-VIS spectrophotometer. Previously, this method was successfully used for the *in vitro* antioxidant activity tests with several herbs (Kadifkova-Panovska *et al*. 2005), sweet potato crude extracts (Huang *et al*. 2004), and it has found many other applications.

The antioxidant activity of food is also frequently characterised by the ferric reducing power (FRP) method, in which the ability of antioxidants present in the sample to reduce the Fe(III) ions to Fe(II) is monitored. The reduction process is accompanied with the development of intensive blue colour, detectable by the monitoring of the absorbance band at 700 nm using UV-VIS spectrophotometer. The higher the absorbance, the higher the reducing power ability. Huang and co-workers used this method for the characterisation of the antioxidant status of sweet potatoes as well as of spinach extracts (Huang *et al*. 2004, 2005, 2006). The antioxidant properties of anthocyanins extracted from litchi were, for instance, also tested with this method (Duan *et al*. 2007).

Folin-Ciocalteau method is routinely used to measure the content of total phenolic compounds (TPC) in various biological systems. It is based on the reduction of metal oxides by polyphenols resulting in a blue solution that has the absorption maximum at 765 nm. Since different types of polyphenols react similarly with the Folin-Ciocalteau reagent, it is quite easy to use it as a quantitative method. The standard curve is in such case usually constructed using gallic or tannin acid and is reported as gallic and tanin acid equivalents, respectively. This method was originally designed to measure phenolic content in wine, but it has also been used to analyse teas, wines, vegetables, and fruit as well as trees and fresh water (Singleton *et al*. 1999; Waterhouse 2001; Thoss *et al*. 2002; Valdez 2002).

The influence of gamma-irradiation on antioxidant ability of food

Several recently published contributions deal with the study of the influence of irradiation procedures on the antioxidant activity of herbs and spices or their extracts. The effects of gamma-irradiation treatment on the free radical and antioxidant contents in nine aromatic herbs and spices (basil, bird pepper, black pepper, cinnamon, nutmeg, oregano, parsley, rosemary, and sage) were studied by Calucci *et al*. (2003). As they concluded, the irradiation at the dose of 10 kGy resulted in a general increase of the quinone radical content in all samples investigated, as revealed by EPR spectroscopy, and in a significant decrease of total ascorbate and carotenoids contents of some spices. The effect of irradiation on the antioxidant properties of seven dessert spices (anise, cinnamon, ginger, licorice, mint, nutmeg, and vanilla) was evaluated by Murcia *et al*. (2004). The results obtained in various radical-scavenging assays proved that water extracts of spices irradiated at the doses from 1 to 10 kGy did not reflect any sizeable changes in their antioxidant activities comparing to those of the respective reference (non-irradiated) spices. The antioxidant properties of anise, caraway, cumin, and fennel essential oils extracted from the untreated samples and from the respective samples treated with gamma-irradiation as well as by microwave absorption were evaluated by Farag and Khawas (1998). As they found, gamma-irradiation at the doses up to 10 kGy and the microwave treatment did not affect the antioxidant properties of the essential oils under study. Sun-dried and dehydrated paprika (*Capsicum annuum* L.) samples were irradiated at the doses from 2.5 to 10 kGy and the capsaicinoid contents were subsequently analysed by Topuz and Ozdemir (2004). The relative contents of

capsaicin, dihydrocapsaicin and homodihydrocapsaicin significantly increased (about 10% in sample treated at dose of 10 kGy).

It was previously confirmed by Pokorny and co-workers that antioxidants in the plant material may act synergistically. Moreover, as they found, leafy spices like thyme, marjoram, basil, sage, or summer savory showed pro-oxidative activity if they were present in foods exposed to light or other specific oxidation stress inducing conditions; while the same food revealed the expected antioxidative effect of the spice presence when stored in the dark (Pokorny *et al*. 2001).

The antioxidant activity of oregano ethanol extract and its inhibitory oxidation effect on the model lipid system prepared from refined bleached peanut oil was tested by Bendini *et al*. (2002).

Oregano contains numerous well described effective antioxidants. Derivatives of phenolic acids, flavonoids, tocopherols, rosmarinic acid, carvacrol, and thymol are the most significant of them (Peter 2000; Pizzale *et al*. 2002; Ruberto *et al*. 2002). According to phytochemical database (USDA 2003), the number of different antioxidants in this spice reaches up to 34.

For the extraction and isolation of the individual antioxidants from oregano, a range of separation techniques and procedures were used, including trichloracetic acid, acetone, methanol, and ethanol extractions, respectively (Jun *et al*. 2001; Exarchou *et al*. 2002; Pizzale *et al*. 2002; Calucci *et al*. 2003), mechanical medium-chain triglyceride and propylene glycol extractions (Schwarz *et al*. 2001), homogenisation in phosphate buffer and subsequent centrifugation (Zheng & Wang 2001), super fluid extraction under specific operating conditions (Nguen *et al*. 1991), or Soxhlet extraction with pure methanol (Ayar *et al*. 2001). The extracts were then tested for the antioxidant properties choosing one of the assays described above or their proper modifications.

The main aim of the investigation presented was the study of the character of the radical species produced upon gamma-radiation treatment of oregano samples using EPR spectroscopy. The influence of the absorbed dose on the character of EPR spectra, as well as the thermal stability and life-time of the gamma-radiation induced radicals were studied. Additionally, the antioxidant properties of the irradiated oregano samples were investigated by means of several antioxidant testing methods. Their changes resulting from the gamma-radiation dose absorption as well as from the subsequent storage were monitored using oregano methanol/water extracts.

MATERIALS AND METHODS

Solid sample characterisation. Commercial ground oregano from Cambidi (Izmir, Turkey) was used in all EPR experiments and for the extracts preparation. The samples were packed into 75 g polyethylene bags and irradiated according to the commercial practices of Artim (Prague, Czech Republic), applying ^{60}Co source at various average doses from 5 up to 30 kGy, using average gamma-radiation dose rate, 2 kGy/h, on June 10, 2004. The average dry matter content determined immediately after irradiation was 90.1%; it increased slightly to 90.6% (w/w) after four months of storage.

Oregano extracts preparation. Oregano extracts used for the determination of the antioxidant activity were prepared as follows: 2 g of respective solid oregano was mixed with 50 ml 80% (v/v) water/methanol solution and the suspension was shaken for 1 hour using a laboratory shaker (Innova 2000, USA) at 200 rpm. The solid phase was separated using filtration and the final extract was stored in a closed box in darkness at ambient temperature (25°C) and relative humidity of 40%.

EPR measurements. Each oregano sample (100 mg) was placed in a thin-wall quartz EPR tube (internal diameter of 3 mm, length of 150 mm, and wall thickness about 0.1 mm) in order to produce a cylindrically shaped sample with uniform dimensions (sample column height 5.2 ± 0.2 cm). The sample was then inserted into a standard TE_{102} (ER 4102 ST) rectangular cavity of an EMX X-band EPR spectrometer (Bruker, Germany) and EPR spectrum was recorded at various temperatures. Temperature control was achieved using a Bruker temperature control unit ER 4111 VT. The careful filling procedure of EPR cells resulted in a good reproducibility between samples with a standard deviation in the relative EPR intensity of ± 5% for five independent measurements. Typical EPR spectrometer settings were as follows: microwave frequency 9.45 GHz; microwave power 0.63–31.73 mW; center field 335.4 mT; sweep width 20–500 mT; gain 5×10^5; modulation amplitude 0.05 mT; modulation frequency 100 kHz; scan 84 s; time constant 40.96 ms; number of scans 5; temperature 298–373 K. The g-values were determined with the uncertainty of ± 0.0005 by the

simultaneous measurement of a reference sample containing DPPH fixed on EPR cell. The EPR instruments settings for the quantitative evaluation were examined by DPPH standard.

The experimental EPR spectra processing and simulation were carried out using WIN EPR and SimFonia programs (Bruker). The integral intensities of the EPR signals were obtained by the double integration of the spectrum. The multi-component experimental EPR spectra were evaluated as a linear combination of the individual EPR spectra simulations using the least-squares minimisation procedure with the Scientist Program (MicroMath). The statistical parameters of the calculation procedure (R^2, coefficient of determination and correlation) served as a determination of the simulation quality, i.e. the correlation of the experimental and simulated spectra. The relative concentrations of the individual paramagnetic species were evaluated from the contributions of the individual simulations to the experimental spectrum after the double integration. They were used for the assessment of the thermal stability and life-time of the individual identified radical structures.

Spectrophotometric measurements. Double-beam UV-VIS spectrometer Specord M40 (Carl Zeiss Jena, Germany) with an appropriate equipment was used for the monitoring of the antioxidant properties. All experiments were carried out in the same square quartz UV-VIS transparent cells (path length, 1 cm).

The monitoring of oregano extract antioxidant ability was realised under the following conditions: spectral bandwidth 20 cm^{-1}; integration time 1 s; gain 3. The series of three independent simultaneous measurements of absorbance for each oregano extract in the respective experimental system were realised and averaged values of absorbance were used for the antioxidant behaviour characterisation. For the statistical significance determination, one factor ANOVA test at the significance level of 0.05 was used.

DPPH radical scavenging assay. DPPH radical scavenging assay was performed according to Bandonienė (2002). Oregano methanol/water extract (0.65 ml) was placed into 25 ml of DPPH methanolic solution $c_0(\text{DPPH}) = 6 \times 10^{-5}$ mol/dm^3, and the absorbance at 515 nm was measured after 15 min. Radical scavenging ability of the individual extracts was expressed as:

% = (absorbance of control – absorbance of sample) × 100/absorbance of control.

Thiobarbituric acid number. Thiobarbituric acid reactive substances were determined according to the method published by Zin (2002). To 1 ml of oregano methanol/water extract, 20% (w/w) aq. trichloroacetic acid (2 ml) and 0.67% (w/w) aq. solution of thiobarbituric acid (2 ml) were added. This mixture was then placed in a boiling water bath for 10 min. After cooling to ambient temperature, the mixture was centrifuged at 3000 rpm for 20 minutes. Thiobarbituric acid number was determined as the absorbance of the supernatant at 532 nm and related to the absorbance of the blank.

Ferric reducing power. The determination of ferric reducing power was realised according to Chyau *et al.* (2002). Oregano methanol/water extract (2 ml) was mixed with 2 ml of 0.2M sodium phosphate buffer (pH 6.6) and 2 ml of 1% (w/w) potassium ferricyanide. The mixture was incubated at 50°C for 20 minutes. Subsequently, 2 ml of 10% (w/w) trichloroacetic acid was added and the mixture was centrifuged at 3000 rpm for 10 minutes. Then 1 ml of the upper layer was mixed with 1 ml of distilled water and 0.2 ml of 0.1% (w/w) ferric chloride. The absorbance at 700 nm was monitored after 1 min and related to the absorbance of the blank.

Total phenolic compounds. The content of total phenolic compounds was determined using the Folin-Ciocalteau modified method (Chaovanalikit & Wrolstad 2004). 100 µl of the methanolic oregano extracts was diluted with 15.9 ml of distilled water and 1 ml of Folin-Ciocalteau reagent (Merck, Germany) was added. After 3 min, 3 ml of 20% of sodium carbonate was added and the content was mixed. As the result of the reaction, a colour was developed and the absorbance at 755 nm was measured after 60 min and related to the absorbance of the blank. The same procedure was repeated using a standard solution of gallic acid. The results were expressed as mg gallic acid/litre of extract.

RESULTS AND DISCUSSION

EPR investigation

Firstly, the EPR spectra of all oregano samples investigated were measured using magnetic field of 500 mT (Figure 1). The observed spectra demonstrated the presence of a broad EPR signal with unresolved hyperfine splittings in all samples.

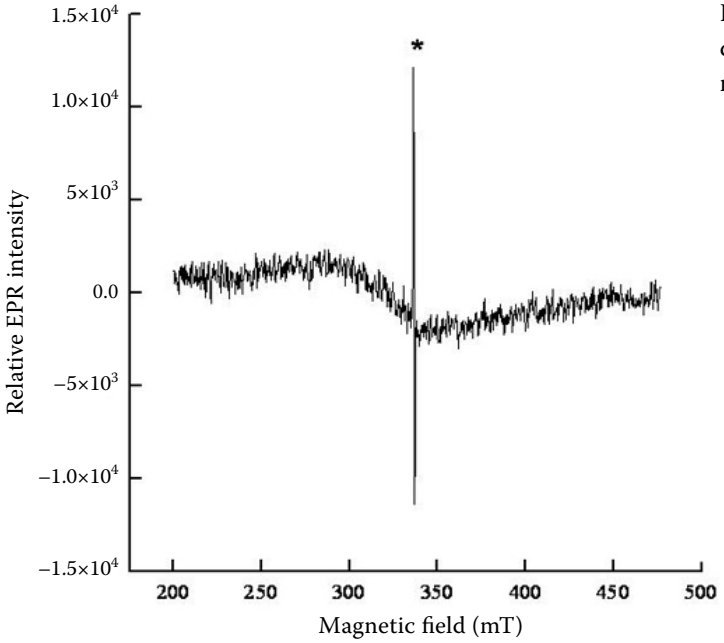

Figure 1. X-band EPR spectrum of non-irradiated dry oregano sample measured using 0.633 mW microwave power at 298 K

Oregano is of plant origin, consequently, this line was attributed to paramagnetic Mn(II) ions as previously mentioned by Morsy and Khaled (2001, 2002); Lozak *et al.* (2002); Morsy (2002); Polat and Korkmaz (2003); Polovka *et al.* (2003, 2006) and Suhaj *et al.* (2006).

Manganese ions play a significant role in biochemical processes of green plants as cofactors of proteins and enzymes. Moreover, they represent an essential component in the catalytic splitting of water and in the evolution of oxygen in the photosystem(II). The expected characteristic feature of the Mn(II) EPR spectra is a six-line component centered at $g_{eff} \approx 2.0$, flanked by shoulders with a weak feature centered at $g_{eff} \approx 4.3$ and a measurable absorption at zero field. The six-line multiplet spectrum results from the hyperfine interaction of the $^6S_{5/2}$ ground state with the ^{55}Mn nucleus (I = 5/2) (Griscom 1990).

We have previously proved (Polovka *et al.* 2003) that relatively undisturbed bonding of Mn(II) in the protein complex is responsible for the less resoluted EPR spectrum of solid tea samples. As we suggest, the same phenomenon influence the shape of EPR spectra of solid oregano samples. Additionally, a characteristic narrow EPR signal (g = 2.0022, $\Delta B_{pp} \sim$ 1 mT), assigned to stable radical structures (Morsy & Khaled 2001, 2002; Morsy 2002; Polovka *et al.* 2003, 2006; Suhaj *et al.* 2006) (* in Figure 1) is superimposed on this broad line. The relative EPR intensity of this sharp signal is significantly dependent on the absorbed gamma-radiation dose.

The detailed simulation analysis of experimental EPR spectra (20 mT magnetic field sweep width) of the reference oregano sample and of the sample irradiated at the dose of 30 kGy is depicted in Figure 2. The spectra of the non-irradiated sample was simulated as a sharp singlet line characterised by $g_{\perp} = 2.0044$, $g_{||} = 2.0010$, and $\lambda\lambda_{pp} = 0.285$ mT which can be attributed to semiquinone radicals produced by the oxidation of polyphenolic compounds present in plants (Morsy & Khaled 2001, 2002; Jezierski *et al.* 2002; Pedersen 2002; Polovka *et al.* 2003, 2006; Ukai & Shimoyama 2003; Suhaj *et al.* 2006).

The application of gamma-radiation on oregano samples led to the formation of additional EPR signals – (*i*) anisotropic triplet ($A_{\perp} = 0.85$ mT, $A_{||} = 0.7$ mT; $g_{\perp} = g_{||} = 2.0061$; $\lambda B_{pp} = 0.67$ mT) attributed to carbohydrate radical structures (Vanhaelewyn *et al.* 2000; Korkmaz & Polat 2001) and (*ii*) anisotropic triplet ($A_{\perp} = 3.0$ mT, $A_{||} = 1.8$ mT; $g_{\perp} = 2.0060$, $g_{||} = 2.0050$; $\lambda B_{pp} = 1.2$ mT previously attributed to "cellulose" radical species (Yordanov *et al.* 1998; Yordanov & Gancheva 2000; Kispéter *et al.* 2003; Bayram & Delincée 2004; Yordanov & Aleksieva 2004). However, the EPR signal of "cellulose" radical species, which the European Committee for Standardization (CEN) in EN 1787:2000 declared as a marker of gamma-irradiation of natural cellulose-containing materials, is minimal (CEN 2000. EN 1787).

Figure 3 shows the dependence of integral EPR intensity of oregano samples on the absorbed

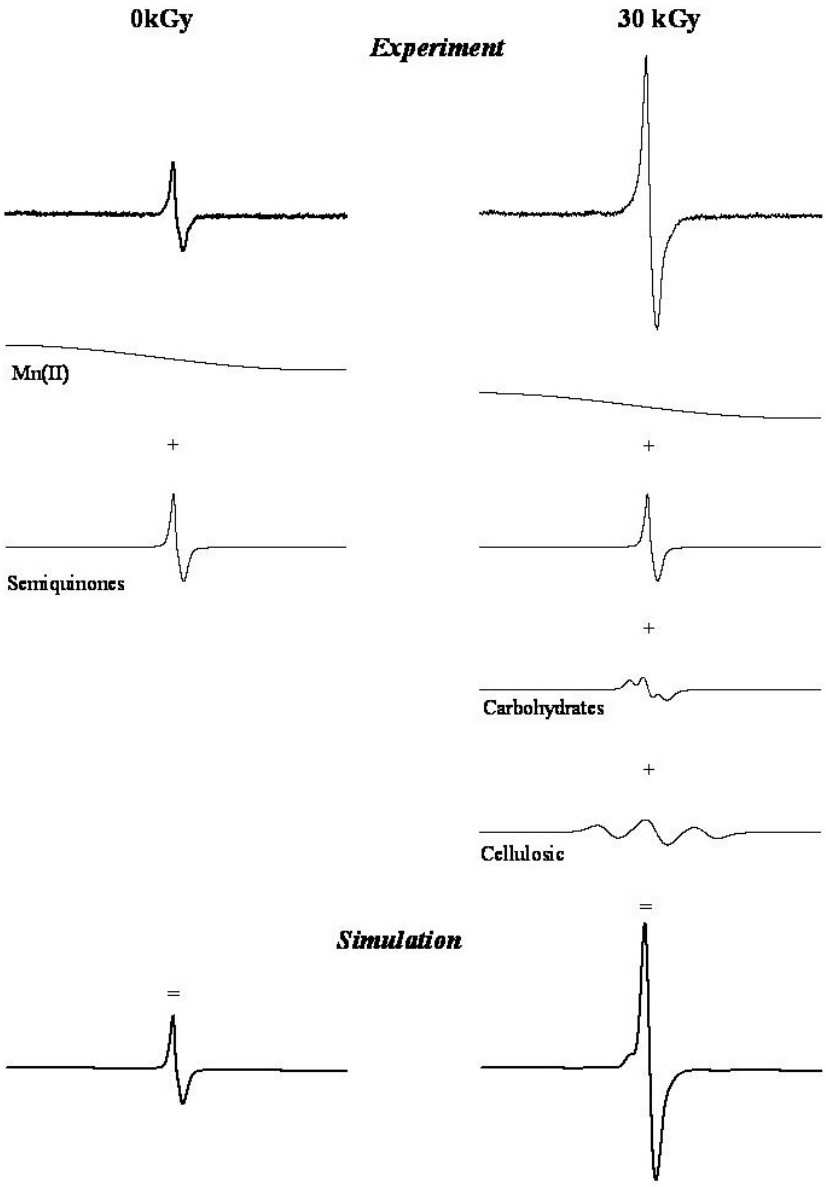

Figure 2. Scheme demonstrating the simulation analysis of experimental EPR spectra of reference (non-irradiated) oregano sample and of sample gamma-irradiated at dose of 30 kGy

Spectra recorded 1 week after irradiation using 0.633 mW microwave power at 298 K; simulation parameters are given in text (R^2 = 0.989)

dose of gamma-radiation, evaluated from experimental EPR spectra measured at different times after the radiation treatment, i.e., one week, 5 and 10 months, respectively. As it is clear, the integral EPR intensity of gamma-radiation induced radical species is significantly related to the gamma-radiation dose, and can be well fitted using the model of saturation curve. On the other hand, it is also demonstrated that the lifetimes of gamma-radiation induced radicals are limited, as a substantial decline of integral EPR intensity was observed, in good agreement with previously published data on gamma-radiated plant materials (Yordanov *et al*. 1998; Yordanov & Gancheva 2000; Kispéter *et al*. 2003; Bayram & Delincée 2004; Yordanov & Aleksieva 2004; Polovka *et al*. 2006; Suhaj *et al*. 2006). We have found that the storage interval after the radiation treatment influenced only the EPR spectra of the irradiated samples, while that of reference remained unchanged. Moreover, the simulation of the experimental spectra showed that the decrease of EPR spectra intensity on storage time for the radiation-induced signals can be described by a formal first-order kinetic model, similar to previously done experiments with black pepper, allspice or other irradiated spices. The individual half-lives calculated confirmed the lowest stability of 'cellulosic' radical structures followed by 'carbohydrates', while the stability of semiquinines remains unaffected by the post-ir-

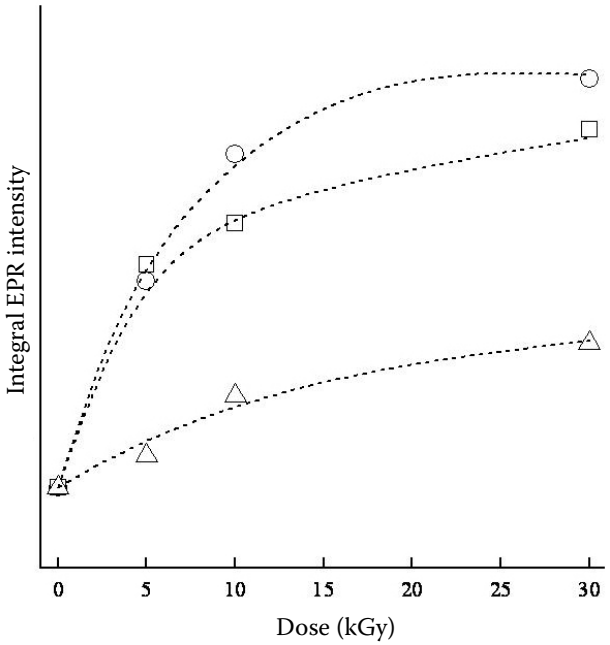

Figure 3. The dependence of integral EPR intensity of oregano samples on gamma-radiation dose evaluated for EPR spectra measured at 298 K using 0.633 mW microwave power one week (○), 5 months (□) and 10 months (△) after irradiation

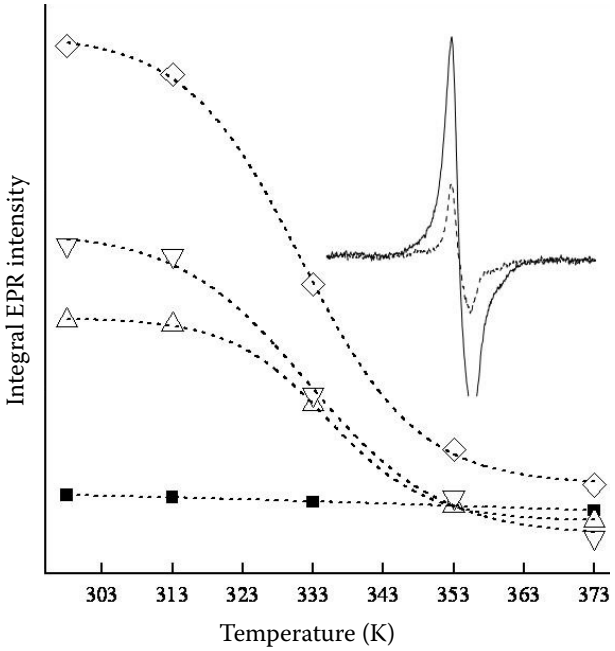

Figure 4. The dependence of integral intensity of EPR spectra of oregano samples on temperature measured at 0.633 mW microwave power one week after γ-irradiation for reference (non-irradiated) sample (■), and sample with adsorbed dose: 5 kGy (△), 10 kGy(▽), 30 kGy (◇)

Inset represents the experimental EPR spectra of sample irradiated at dose 30 kGy, measured at 298 K (–) and at 373 K(•••), respectively. Magnetic field width 8 mT (EPR spectra were recorded 1 week after gamma-radiation treatment)

radiation storing. These results correspond well with previously published data (Raffi *et al*. 2000; Delincée 2002; Polovka *et al*. 2006; Suhaj *et al*. 2006).

The stability of the radiation-induced radicals is also strongly temperature-dependent. As depicted in Figure 4, the EPR spectra of the reference sample showed only negligible changes with increasing temperature from 298 K to 353 K. On the contrary, the behaviour of the irradiated samples under increasing temperature is quite different. The rise in temperature from 298 K to 353 K caused a significant and irreversible decrease of gamma-radiation induced signals. The study of the thermal stability of EPR signal can be useful in the prediction of γ-irradiation treatment of cellulose-containing samples, as recommended by Yordanov and Gancheva (2000). As we have found from the detailed simulation of the experimental EPR spectra, the decay of each individual radical structure can be well fitted to the model of Arrhenius equation and the effective parameters so obtained can be used for the radical thermal stability assessment. Cellulosic radicals possess the lowest thermal stability, and have also the lowest life-time, followed by carbohydrate radical structures, while the stability of semiquinones remains almost unaffected by the thermal treatment. These conclusions correlate well with our previously published results obtained with black pepper treated by gamma-irradiation (Polovka *et al*. 2006; Suhaj *et al*. 2006).

Antioxidant activity of oregano extracts

Antioxidant properties of irradiated oregano samples were investigated using several antioxidant testing methods. The influence of irradiation and subsequent storage of oregano samples on the DPPH radical-scavenging ability was only negligible. The same conclusion was made by Murcia *et al*. (2004) in the investigation of the antiradical activity of dessert spices. Similar results were obtained in the evaluation of the influence of gamma-irradiation on ferric reducing power. The FRP values of the extract prepared from the sample treated by 30 kGy

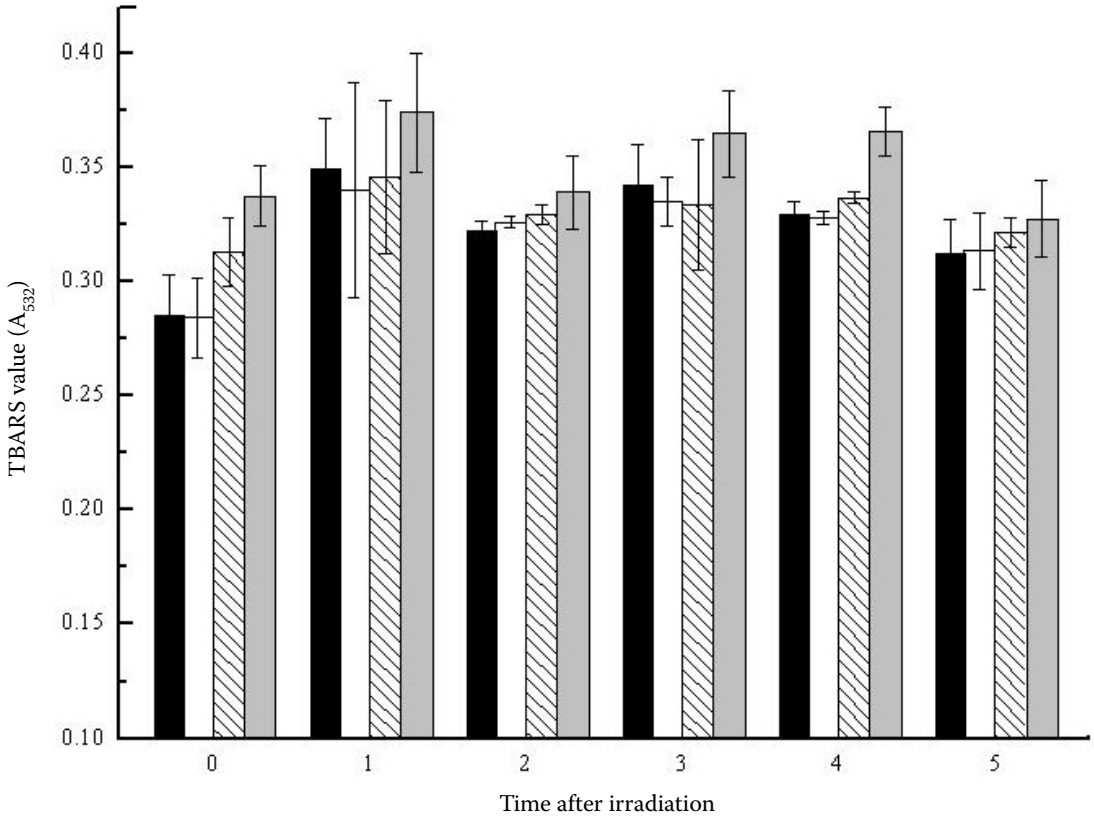

Figure 5. Effect of irradiation and storage time on thiobarbituric reactive substances content (TBARS) of oregano methanolic extracts prepared from reference sample (■) and from oregano samples irradiated at 5 kGy (□), 10 kGy (▨), and 30kGy (■)

was undistinguishable from those of reference or the sample irradiated at lower doses. Post-irradiation storage caused only minor changes in FRP values, only ferric reducing ability of the sample irradiated at 5 kGy monitored after 4 months of storage was slightly increased, i.e. by about 16% compared to the values determined immediately after the irradiation. (Data not shown.)

On the other hand, a significant dependence of oregano extracts TBARS values on the absorbed dose of gamma-radiation was found. As shown in Figure 5, the irradiation caused a considerable increase of TBARS values of oregano extract prepared from the sample irradiated at 30 kGy – about 18% compared to that prepared from the reference sample immediately after the radiation process. This difference remains throughout the 5 months storage. Moreover, the one month post-irradiation storage resulted in a statistically significant increase of TBARS values in all oregano sample extracts. The most significant difference reached approx. 20%. The continuous storage leads to a slight decrease of all TBARS values with the time.

In the case of total phenolic compounds expressed as gallic acid equivalent (Figure 6), statistically significant changes were found resulting from the radiation treatment and post-irradiation storage of oregano. The TPC content in the reference sample reached maximum after one month of storage and thereafter it gradually decreased. The changes in the extracts prepared from reference are closely related to the dry matter content alterations with the time.

Gamma-radiation treatment caused a conspicuous increase of TPC in all oregano extracts. The TPC values of the extracts prepared from the samples irradiated at lower doses (up to 10 kGy) slowly rose with the absorbed dose of gamma-radiation, reaching the maximum in the extract prepared from the sample treated by 10 kGy. On the other hand, in the extracts prepared from the samples irradiated at 20 kGy and 30 kGy, the decline of TPC values was noticed. One-month post-irradiation storage caused a minor growth (about 10%) of polyphenolic content in all extracts. The differences between the samples diminished

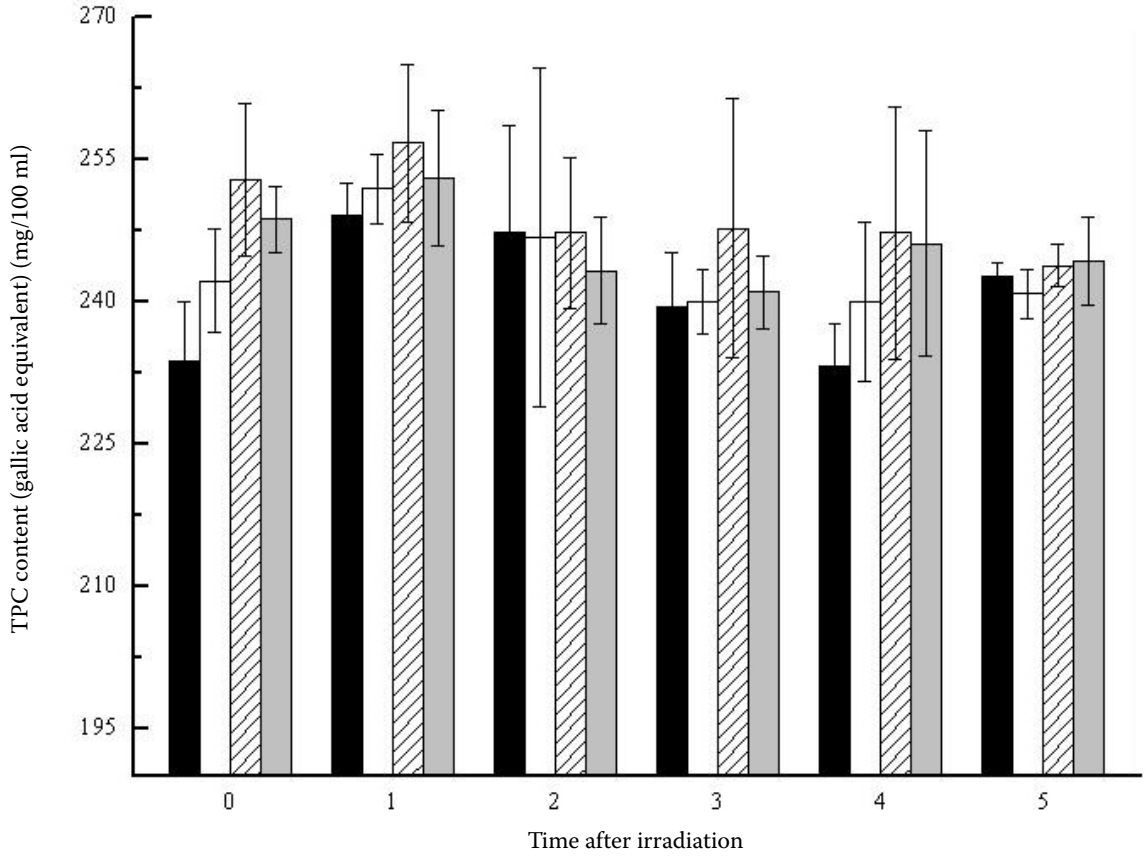

Figure 6. Effect of irradiation and storage time on total phenolic content (TPC) of oregano methanolic extracts. TPC values are expressed as gallic acid equivalent. Extracts were prepared from reference sample (■) and from oregano samples irradiated at 5 kGy (□), 10 kGy (▨), and 30kGy (■)

after 5 months of storage. The absolute values of TPC decreased during the storage as well, but at the end of the investigation period they were still higher compared to that determined immediately after the irradiation. The above discussed effect of irradiation on phenolics content is in a good agreement with the conclusions of several recently published papers (Oufedjikh *et al*. 2000; Vanamala *et al*. 2003).

CONCLUSIONS

EPR spectroscopy confirmed that gamma-radiation treatment of oregano samples resulted in a dose-dependent generation of paramagnetic species of different structures, attributed to the cellulosic and carbohydrate radicals. Their stability is significantly affected by temperature, relative humidity, and storage conditions. The cellulosic radical structures showed both the worst thermal stability and life-time as compared to carbohydrates as well as to semiquinones found in the reference sample. All these factors have to be taken into account in order to survey the changes induced in spices by ionising radiation absorption, and to use EPR spectroscopy as a valuable dosimetric method.

The irradiation process influenced only in a negligible way both the ability of oregano extract to terminate DPPH free radicals and to reduce ferric ions to ferrous. Slightly increased TBARS values and total phenolic content were observed in the extracts prepared from the irradiated samples immediately after the radiation process. As we have found, after 5 months of post-irradiation storage the differences in TBARS values still persist, while the TPC content differences gradually disappeared.

Acknowledgement. Referees are gratefully acknowledged for their valuable comments.

References

Antolovich M., Prenzler P.D., Patsalides E., McDonald S., Robards K. (2002): Methods for testing antioxidant activity. Analyst, **127**: 183–198.

Ayar A., Oczan M., Akgul A., Akin N. (2001): Butter stability as affected by extracts of sage, rosemary and oregano. Journal of Lipids, **8**: 15–25.

Bandonienė D., Murkovic M., Pfanhauser W., Venskutonis P.R., Gruzdiéne D. (2002): Detection and activity evaluation of radical scavenging compounds by using DPPH free radical and on-line HPLC-DPPH methods. European Food Research and Technology, **214**: 143–147.

Bayram G., Delincée H. (2004): Identification of irradiated Turkish foodstuffs combining various physical detection methods. Food Control, **15**: 81–91.

Bendini A., Toschi T.G., Lercker G. (2002): Antioxidant activity of oregano leaves. Italian Journal of Food Science, **14**: 17–25.

Calenberg S., Vanhaelewyn G., Cleemput O., Callens F., Mondelaers W., Huyghebaert A. (1998): Comparison of the effect of X-ray and electron beam irradiation on some selected spices. Lebensmittel-Wissenschaft und -Technologie, **31**: 252–258.

Calucci L., Pinzono C., Zandomeneghi M., Capocchi A. (2003): Effects of γ-irradiation on the free radical and antioxidant contents in nine aromatic herbs and spices. Journal of Agricultural and Food Chemistry, **51**: 927–934.

CEN 2000. EN 1787, Determination of irradiated food containing cellulose: analysis by EPR. European Committee for Standardization, Brussels.

Chabane S., Pouliquen-Sonaglia I., Raffi J. (2001): Detection of irradiated spices by different physical techniques. Canadian Journal of Physiology & Pharmacology/Review of Canadian Physiology & Pharmacology, **79**: 103–108.

Chaovanalikit A., Wrolstad R.E. (2004): Total anthocyanins and total phenolics of fresh and processed cherries and their antioxidant properties. Journal of Food Science, **69**: 67–72.

Chyau C.C., Tsai S.Y., Ko P. T., Mau J.L. (2002): Antioxidant properties of solvent extracts from *Terminalia catappa* leaves. Food Chemistry, **78**: 483–488.

Code of Federal Regulation: 21CFR179: Irradiation in the Production, Processing and Handling of Food. Title 21, Volume 3. Revised as of April 1, 2004.

Codex Stan 106 – 1983, Rev. 1 – 2003: Codex General Standard for Irradiated Foods. Food and Agriculture Organisation of the United Nations, Rome

Delincée H. (2002): Analytical methods to identify irradiated food – a review. Radiation Physics and Chemistry, **63**: 455–458.

Directive 1999/3/EC of the European Parliament and of the Council of 22 February 1999 on the establishment of a Community list of foods and food ingredients treated with ionising radiation. Official Journal of the European Communities, 1999, L 66: 24.

Duan X., Jiang Y., Su X., Zhang Z., Shi J. (2007): Antioxidant properties of anthocyanins extracted from litchi (*Litchi chinenesis* Sonn.) fruit pericarp tissues in relation to their role in the pericarp browning. Food Chemistry, **101**: 1365–1371.

European Standard EN 13708 (2001): Detection of irradiated food containing crystalline sugar by ESR spectroscopy. European Committee for Standardization, Brussels.

European Standard EN 13751 (2002): Detection of irradiated food using photostimulated luminescence. European Committee for Standardization, Brussels.

European Standard EN 13784 (2001): DNA comet assay for the detection of irradiated foodstuffs – Screening method. European Committee for Standardization, Brussels.

European Standard EN 1784 (1996): Detection of irradiated food containing fat – Gas chromatographic analysis of hydrocarbons. European Committee for Standardization, Brussels.

European Standard EN 1785 (1996): Detection of irradiated food containing fat – Gas chromatographic/mass spectrometric analysis of 2- alkylcyclobutanones. European Committee for Standardization, Brussels.

European Standard EN 1786 (1996): Detection of irradiated food containing bone – Method by ESR spectroscopy. European Committee for Standardization, Brussels.

European Standard EN 1787 (2000): Determination of irradiated food containing cellulose: Method by ESR Spectroscopy. European Committee for Standardization, Brussels.

Exarchou V., Nenadis N., Tsimidou M., Gerothanassis I.P., Troganis A., Boskou D. (2002): Antioxidant activities and phenolic composition of extracts from Greek oregano, Greek sage, and summer savory. Journal of Agricultural and Food Chemistry, **50**: 5294–5299.

Farag R.S., Khawas K.H. (1998): Influence of gamma-irradiation and microwaves on the antioxidant property of some essential oils. International Journal of Food Sciences and Nutrition, **49**: 109–115.

Formanek Z., Barabássy S., Chabane S., Molina C., Deyris V. (1999): Identification of irradiation in coded black pepper samples by different physical methods (viscosimetry and ESR). Acta Alimentaria, **28**: 103–109.

Franco R.W.A., Neto L.M., Kato M.S.A., Furlan G.R., Walder J.M.M., Colnago L.A. (2004): Identification of irradiation treatment in black pepper by electron paramagnetic resonance. International Journal of Food Science & Technology, **39**: 395–401.

Griscom D.L. (1990): Electron spin resonance. In: Uhlmann D.R., Kriedl N.J. (eds): Glass. Science and Technology, 4B. Academic Press, Boston: 151–170.

Huang D.-J., Lin Ch.-D., Chen H.-J., Lin Y.-H. (2004): Antioxidant and antiproliferative activities of sweet potato (*Ipomoea batatas* [L.]) constituents. Botanical Bulletin of Academia Sinica, **45**: 179–186.

Huang D.-J., Chen H.-J., Lin Ch.-D., Lin Y.-H. (2005): Antioxidant and antiproliferative activities of water spinach (*Ipomoea aquatica* Forsk) constituents. Botanical Bulletin of Academia Sinica, **46**: 99–106.

Huang D.-J., Chen H.-J., Hou W.-Ch., Lin Ch.-D., Lin Y.-H. (2006): Sweet potato (*Ipomoea batatas* [L.] Lam 'Tainong 57') storage root mucilage with antioxidant activities *in vitro*. Food Chemistry, **98**: 774–781.

Jezierski A., Czechowski F., Jerzykiewicz M., Golonka I., Drozd J., Bylinska E., Chen Y., Seaward M.R.D. (2002): Quantitative EPR study on free radicals in the natural polyphenols interacting with metal ions and other environmental pollutants. Spectrochimica Acta A, **58**: 1293–1300.

Jun W.J., Han B.K., Yu K.W., Kim M.S. (2001): Antioxidant effects of *Origanum majorana* L. on superoxide anion radicals. Food Chemistry, **75**: 439–444.

Kadifkova-Panovska T., Kulevanova S., Stefova M. (2005): *In vitro* antioxidant activity of some *Teucrium* species (*Lamiaceae*). Short communication. Acta Pharmacologica, **55**: 207–214.

Kispéter J., Bajúsz-Kabók K., Fekete M., Szabó G., Fodor E., Páli T. (2003): Changes induced in spice paprika powder by treatment with ionizing radiation and saturated steam. Radiation Physics and Chemistry, **68**: 893–900.

Korkmaz M., Polat M. (2001): Radical kinetics and characterization of the free radicals in gamma irradiated red pepper. Radiation Physics and Chemistry, **62**: 411–424.

Lozak A., Soltyk K., Ostapczuk P., Fijalek Z. (2002): Determination of selected trace elements in herbs and their infusions. Science of the Total Environment, **289**: 33–40.

Morsy M.A. (2002): Teas: Direct test on quality and antioxidant activity using electron paramagnetic resonance spectroscopy. Spectroscopy, **16**: 371–378.

Morsy M.A., Khaled M.M. (2001): Direct electron paramagnetic resonance study of tobacco. 1. Manganese(II) as a marker. Journal of Agricultural and Food Chemistry, **49**: 683–686.

Morsy M.A., Khaled M.M. (2002): Novel EPR characterization of the antioxidant activity of tea leaves. Spectrochimica Acta A, **58**: 1271–1277.

Murcia M.A., Egea I., Romojaro F., Parras P., Jimenez A.M., Martinez-Tome M. (2004): Antioxidant evaluation in dessert spices compared with common food additives. Influence of irradiation procedure. Journal of Agricultural and Food Chemistry, **52**: 1872–1881.

Nguen U., Frakman G., Evans D.A. (1991): Process for extracting antioxidants from *Labiatae* herbs. United States Patent 5 017 397, May 21, 1991.

Oufedjikh H., Mahrouz M., Amiot M.J., Lacroix M. (2000): Effect of gamma-irradiation on phenolic compounds and phenylalanine ammonia-lyase activity during storage in relation to peel injury from peel of *Citrus clementina* Hort. ex. Tanaka. Journal of Agriculture and Food Chemistry, **48**: 559–565.

Pedersen J.A. (2002): On the application of electron paramagnetic resonance in the study of naturally occurring quinones and quinols. Spectrochimica Acta A, **58**: 1257–1270.

Peter K.V. (2000): Handbook of herbs and spices. CRC Press, Woodhead Publishing Lim., Cambridge: 319.

Pizzale L., Bortolomeazzi R., Vichi S., Conte L.S. (2002): Antioxidant activity of sage and oregano extracts related to their phenolic compound content. Journal of the Science of Food and Agriculture, **82**: 1645–1651.

Pokorny J., Yanislieva N., Gordom M. (2001): Antioxidants in Food – Practical Applications. Woodhead Publishing Lim., CRC Press, Boca, Raton, Boston, New York, Washington: 380.

Polat M., Korkmaz M. (2003): The effect of temperature and storage time on the resonance signals of irradiated pea (*Pisum sativum* L.). Food Research International, **8**: 857–862.

Polovka M., Brezová V., Staško A. (2003): Antioxidant properties of tea investigated by EPR spectroscopy. Biophysical Chemistry, **106**: 39–56.

Polovka M., Brezová V., Staško A., Mazúr M., Suhaj M., Šimko P. (2006): EPR investigations of gamma-irradiated ground black pepper. Radiation Physics and Chemistry, **75**: 309–321.

Raffi J., Yordanov N.D., Chabane S., Douifi L., Gancheva V., Ivanova S. (2000): Identification od irradiation treatment of aromatic herbs, spices and fruits by electron paramagnetic resonance and thermoluminescence. Spectrochimica Acta A, **56**: 409–416.

Ruberto G., Baratta M.T., Kaabeche M. (2002): Chemical composition and antioxidant activity of es-

sential oils from Algerian *Origanum glandulosum* Desf. Flavour and Fragrance Journal, **17**: 251–254.

Schwarz K., Bertelsen G., Nissen L.R., Gardner P.T., Heinonen M.I., Hopia A. (2001): Investigation of plant extracts for the protection of processed foods against lipid oxidation. European Food Research and Technology, **212**: 319–328.

Singleton V.L., Orthofer R., Lamuela-Raventos R.M. (1999): Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteau reagent. Methods in Enzymology, **299**: 152–178.

Staško A., Liptáková M., Malík F., Mišik V. (2002): Free radical scavenging activities of white and red wines. An EPR spin trapping study. Applied Magnetic Resonance, **22**: 101–113.

Staško A., Polovka M., Brezová V., Biskupič S., Malík F. (2006): Tokaj wines as scavengers of free radicals (An EPR study). Food Chemistry, **96**: 185–196.

Suhaj M., Rácová J., Polovka M., Brezová V. (2006): Effect of gamma-irradiation on antioxidant activity of black pepper (*Piper nigrum* L.). Food Chemistry, **97**: 696–704.

Thoss V., Bair D.M.S., Locka M.A., Courty P.V. (2002): Quantifying the phenolic content of freshwaters using simple assays with different underlying reaction mechanisms. Journal of Environmental Monitoring, **4**: 270–275.

Topuz A., Ozdemir F. (2004): Influences of gamma irradiation and storage on the capsaicinoids of sun-dried and dehydrated paprika. Food Chemistry, **86**: 509–515.

Ukai M., Shimoyama Y. (2003): An electron spin resonance study of evolution of organic free radicals in irradiated pepper during heat treatment. Journal of Food Science, **68**: 2225–2229.

USDA, ARS, National Genetic Resources Program. Phytochemical and Ethnobotanical Databases. [Online Database.] National Germplast Resources Laboratory, Beltsville, Maryland. Available from http://www.ars-grin.gov/duke – 25 June 2003.

Valdez L.B., Goretta L.A., Boveris A. (2002): Polyphenols in red wines prevent NADH oxidation induced by peroxynitrite. Annals of the New York Academy of Sciences, **957**: 274–278.

Vanamala J.K.P., Cobb B.G., Pike L.M., Patil B.S. (2003): Ionizing radiation and storage effects on postharvest quality of Rio red grapepefruit. Acta Horticulturae, **628**: 111–117.

Vanhaelewyn G., Sadlo J., Callens F., Mondelaers W., De Frenne D., Matthys P. (2000): A decomposition study of the EPR spectrum of irradiated sucrose. Applied Radiation Isotopes, **52**: 1221–1227.

Waterhouse A.L. (2001): Determination of Total Phenolics. In: Wrolstad R.E. (ed.): Current Protocols in Food Analytical Chemistry. John Wiley & Sons, Inc., Weinheim, Germany: Chapts I1.1.1–I1.1.8.

Yordanov N.D. (1996): Is our knowledge about the chemical and physical properties of DPPH enough to consider it as a primary standard for quantitative EPR spectrometry. Applied Magnetic Resonance, **10**: 339–350.

Yordanov N.D., Aleksieva K. (2004): X- and Q-band EPR studies on fine powders of irradiated plants. New approach for detection of their radiation history by using Q-band EPR spectrometry. Radiation Physics and Chemistry, **69**: 59–64.

Yordanov N.D., Gancheva V. (2000): A new approach for extension of the identification period of irradiated cellulose-containing foodstuffs by EPR spectroscopy. Applied Radiation and Isotopes, **52**: 1958–1983.

Yordanov N.D., Gancheva V., Radicheva M., Hristova B., Guelev M., Penchev P. (1998): Comparative identification of irradiated herbs by the methods of electron paramagnetic resonance and thermoluminescence. Spectrochimica Acta A, **54**: 2413–2419.

Zheng W., Wang S.Y. (2001): Antioxidant activity and phenolic compounds in selected herbs. Journal of Agricultural and Food Chemistry, **49**: 5165–5170.

Zin Z.M. (2002): Antioxidative activity of extracts from Mengkudu (*Morina citrifolia* L.) root, fruit and leaf. Food Chemistry, **78**: 227–231.

Received for publication January 24, 2006
Accepted after corrections February 28, 2007

Corresponding author:

Ing. Martin Polovka, Ph.D., Výskumný ústav potravinársky, Priemyselná 4, 824 75 Bratislava 25, Slovenská republika
tel.: + 421 250 237 174, fax: + 421 255 571 417, e-mail: polovka@vup.sk